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13. ABSTRACT *(Maximum 200 words)*

We analyzed 511 (case=156, control 1=184, control 2=171) archived samples of blood from a previous case-control study of breast cancer for two vitamin D metabolites: 25(OH)D and $1,25(OH)_2D$. Our dataindicate that white women at the time of first diagnosis of breast cancer have significantly lower mean levels of $1,25(OH)_2D$ than comparable women without breast cancer. The odds ratio (95% CI) for lowest relative to highest quartile of $1,25(OH)_2D$ was 6.3 (2.6, 15.3) in a comparison of white cases and control 1 subjects, after adjustment for age, month of blood draw, clinic, assay batch, and sample storage time. The risk was stronger in women above the median age of 54, 5.2 (2.4, 11.1), than for younger women, 1.3 (0.7, 2.6). We did not see the same pattern of risk associated with low $1,25(OH)_2D$ among the small number of black women in ourstudy. There were no differences between cases and controls in mean 25(OH)D levels in either race. This work may have important implications for the chemoprevention and treatment of breast cancer.

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Esther C. Jones Esq. April 15 '97

PI - Signature Date

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INTRODUCTION

Recent work has defined a previously unsuspected involvement of vitamin D in cellular growth and differentiation[1,2]. This recognition has fostered an interest in the investigation of a possible role for vitamin D in carcinogenesis[3]. Several cancers have been evaluated for a relationship between tumor occurrence and low levels of vitamin D. There is a suggestion in the literature that decreased intake of vitamin D may be associated with cancer of the colon[4]; blood levels of vitamin D are lower in African-American and white men with cancer of the prostate than in similar men without prostate cancer[5].

Evidence for the possible relationship between vitamin D and breast cancer is based on several lines of investigation. Ecologic studies generally support a relationship between low levels of sunlight exposure and breast cancer[6,7,8]. A single case-control study in Canada, however, failed to demonstrate an association between low levels of vitamin D, as determined by dietary history, and breast cancer[9]. Studies in rats show an increased incidence of mammary tumors under conditions of low dietary vitamin D (0.05 IU/kcal) and calcium (0.25 mg/kcal)[10]. 1,25-dihydroxy vitamin D ($1,25(OH)_2D$), which is the hormonally active metabolite, displays a growth-inhibitory effect on human breast adenocarcinoma cells in culture irrespective of their sex-steroid dependence[11]. Cultures of the human breast cancer cell line BT-20 demonstrate increased differentiation when exposed to $1,25(OH)_2D$ daily for 8 days[12].

The purpose of our current work is to determine whether there are differences in blood levels of 1,25-dihydroxyvitamin D between women with breast cancer and two control groups of women without breast cancer. We are using archived samples of blood obtained from women in a just-completed case-control study of genetic risk factors for breast cancer. In addition, we are working to modify the immunohistochemical technique for detection of vitamin D receptors (VDR) described for frozen tissue for use in paraffin embedded tissue. If we are successful, we will be able to evaluate the VDR status of tumor tissue from our subjects with breast cancer.

The specific aims of our study are as follows: 1) To determine if blood levels of 25-hydroxy vitamin D and 1,25-dihydroxyvitamin D are lower in women at the time of first diagnosis of breast cancer than in comparable women who do not have breast cancer. 2) To describe the relationship between levels of 25-hydroxy vitamin D and 1,25-dihydroxyvitamin D and previously described risk factors for breast cancer. 3) To identify and quantify the distribution of vitamin D receptors in tumor samples from the women diagnosed with breast cancer. 4) To identify the relationship between previously described risk factors for breast cancer and blood levels of 25-hydroxy vitamin D and 1,25-dihydroxyvitamin D with vitamin D receptor (+) and vitamin D receptor (-) breast cancers.

Body

Task 1: Analysis of vitamin D metabolites (0-15) months.

We have completed the analysis of the vitamin D metabolites for the entire group of 511 samples. Our vitamin D assays were done in 13 batches of 40 samples each with an admixture of randomly selected samples consisting of approximately one-third cases and one-third controls from each of the two control groups. Fourteen percent of the samples had to be reassayed because of technical difficulties such as problems with recovery. We used the calf thymus radioreceptor assay from INCSTAR (Stillwater, Minnesota) for determination of $1,25(OH)_2D$ and the radioimmunoassay from INCSTAR (Stillwater, Minnesota) for determination of 25(OH)D. A summary of the mean levels of the precursor metabolite, 25(OH)D, and the active metabolite, $1,25(OH)_2D$ as well as median values and ranges by race are presented in Table 1. 8 Asian-American, 1 Native American, and 5 Hispanic women were excluded from the analyses because their numbers were too small to provide meaningful results.

Table 1. Comparison of vitamin D metabolite levels in cases and controls by race

	Black			White		
	case n =21	control 1 n=30	control 2 n=23	case n=131	control 1 n=149	control 2 n=143
D1,25, pg/ml						
* mean (SD)	23.87 (9.42)	21.42 (8.68)	25.73 (10.45)	18.59 (8.62)	23.53 (9.01)	22.39 (9.02)
median	21.47	20.10	26.80	17.18	22.29	20.63
range	6.71 - 45.16	5.28 - 39.49	6.35 - 44.07	2.71 - 45.29	1.88 - 47.51	4.61 - 50.85
D25, ng/ml						
* mean (SD)	8.91 (6.50)	9.94 (7.40)	11.06 (6.42)	15.06 (8.81)	14.71 (7.41)	13.96 (6.45)
median	6.80	8.125	7.99	13.49	12.98	13.31
range	3.52 - 28.47	1.87 - 40.33	3.81 - 26.73	0 - 48.44	2.14 - 48.40	3.0 - 40.71

*P-value for t test of difference in means:

	Black		White	
D1,25	case versus control 1	0.34	case versus control 1	0.0001
	case versus control 2	0.54	case versus control 2	0.0004
D25	case versus control 1	0.61	case versus control 1	0.72
	case versus control 2	0.28	case versus control 2	0.24

There was a significant difference in $1,25(OH)_2D$ pg/ml between white cases and each control group; there were no differences in mean values of 25(OH)D. There were no

statistically significant differences in either $1,25(OH)_2D$ or 25(OH)D between black cases and either control group.

The assay of the vitamin D metabolites has been completed; in white women, the results support the hypothesis that women with breast cancer have lower levels of $1,25(OH)_2D$ at the time of first diagnosis of their disease than comparable women without breast cancer. We do not see the same relationship between $1,25(OH)_2D$ level and disease risk in the small number of black women in the study.

Task 2: Development of Assay for VDR (0-6 months)

We have made slow progress in developing the assay; we are using human intestine, known to have the VDR, for the development of the assay in our lab. We have been collaborating with Dr. Lynn Dressler in the Molecular Epidemiology Laboratory at the University of North Carolina. At present, we are using streptavidin conjugated to glucose oxidase. This has resulted in a marked decrease in background staining and better color development. After establishing reliable positive and negative controls, we will begin to use the assay in breast tissue.

In preparation for the application of these methods to fixed tissues, we have established a working relationship with Dr. Lester Layfield's laboratory (Department of Pathology, Duke University). In this clinical lab, they have adapted methods for the localization of the estrogen receptor in frozen tissues for use on fixed specimens. Dr. Layfield has offered several protocols for our use and the resources of his laboratory to enable us to successfully adapt the immunohistochemical methods for VDR visualization in frozen specimens for sections of fixed tissue.

Task 3: Detection of VDR's in paraffin-embedded tumor specimens (6-18 months).

We have been unable to proceed with this task because of the difficulties in developing a satisfactory assay for the VDR (see Task 2).

Task 4: Data analysis and report writing (15-24 months).

We have completed analysis of the data relating to Specific Aim 1 of our study: to determine if blood levels of 25(OH)D and $1,25(OH)_2D$ are lower in women at the time of initial diagnosis of breast cancer than in comparable women who do not have breast cancer. Table 2 describes the risk associated with being in various quartiles of $1,25(OH)_2D$ with the highest quartile as the reference. Black subjects did not show the pattern of increased risk associated with low levels of $1,25(OH)_2D$ that was evident in white subjects. Risk levels were highest for the comparison of white cases and control 1 subjects, groups who were matched on month of blood drawing. When adjusted for age, month of blood draw, clinic, assay batch, and sample storage time, the OR (95% CI) for lowest versus highest quartile of $1,25(OH)_2D$ for white cases and control 1 women was 6.3 (2.6, 15.3)

Table 2. Crude Odds ratios (95% CI) for the risk of breast cancer associated with quartile of $1,25(OH)_2D$

		Quartile of $1,25(OH)_2D$ pg/ml*				
	n	1	2	3	4 (ref)	p trend
All subjects	511	2.9 (1.7, 5.1)	1.5 (0.8, 2.7)	1.4 (0.8, 2.6)	1	< 0.0001
Black subjects	74	0.8 (0.2, 3.6)	1.6 (0.4, 6.5)	1.1 (0.3, 4.6)	1	0.96
White subjects	423	3.8 (2.0, 7.0)	1.5 (0.8, 3.1)	1.4 (0.7, 2.8)	1	<0.0001
cases/control 1	280	4.4 (2.2, 8.7)	1.3 (0.6, 2.8)	1.3 (0.6, 2.8)	1	<0.0001
cases/control 2	274	3.4 (1.7, 6.9)	1.6 (0.8, 3.4)	1.8 (0.9, 3.9)	1	0.001

quartiles*:	1	2	3	4
all	≤16.470	16.48 - 21.38	21.39 - 28.04	> 28.04
black	≤16.699	16.70 - 22.07	22.08 - 30.44	>30.44
white	≤16.597	16.60 - 21.62	21.63 - 28.07	>28.07
control 1	≤18.130	18.14 - 22.29	22.29 - 27.94	>27.94
control 2	≤15.720	15.73 - 20.64	20.65 - 28.39	>28.39

*quartile ranges in picograms/ml are based on the distribution of metabolite in the respective control group; values for control groups 1 and 2 are based on white subjects

We have also completed the analysis of the data relating to Specific Aim 2: to describe the relationship between levels of 25(OH)D and $1,25(OH)_2D$ and previously described risk factors for breast cancer. There were no significant associations between either lower or higher levels of 25(OH)D and previously described risk factors for breast cancer such as older age, postmenopausal status, higher BMI, younger age at menarche, or use of hormone replacement therapy among the white control 1 women. $1,25(OH)_2D$ levels were higher in postmenopausal white control 1 women (p=0.046).

We did a stratified analysis with white subjects, cases and control group 1 to investigate possible interactions between $1,25(OH)_2D$ and selected covariates (Table 3).

Table 3. Stratified analysis of breast cancer risk associated with levels of $1,25(OH)_2D$ below the median value of 22.29 pg/ml, white cases and control group 1 (n=280)

Variable	$1,25(OH)_2D$ pg/ml				OR (95% CI)	p*
	≤ 22.29		>22.29			
	case	cont	case	cont		
Age, years						
> 54	50	31	13	42	5.2 (2.4, 11.1)	0.0008
≤ 54	43	43	25	33	1.3 (0.7, 2.6)	
Menopausal status						
post-	64	45	25	57	3.2 (1.8, 5.9)	0.11
pre-	29	29	13	18	1.4 (0.6, 3.3)	
BMI, kg/m^2						
> 24.13	44	32	21	42	2.8 (1.4, 5.5)	0.70
≤ 24.13	49	42	17	33	2.3 (1.1, 4.6)	
25(OH)D, ngm/ml						
≤ 12.98	45	35	19	40	2.7 (1.3, 5.5)	0.78
> 12.98	48	39	19	35	2.3 (1.1, 4.6)	
Hormone replacement therapy						
yes	37	29	16	29	2.3 (1.1, 5.0)	0.85
no	56	45	22	45	2.5 (1.3, 4.8)	

*p value for the Breslow Day χ^2_{1df} test for homogeneity of the stratum specific ORs

An interaction was considered present if the Breslow-Day statistic for homogeneity of the odds ratio was significant a the 0.05 level. The magnitude of breast cancer risk related to low $1,25(OH)_2D$ level was greater for women above the age of 54 than for women 54 and younger ($p=0.0008$). There was no difference in risk according to menopausal status, BMI, 25(OH)D level, or use of hormone replacement therapy.

We have prepared and submitted a manuscript on the results of our study; we are preparing a second manuscript describing the methods used to assess assay validity and reliability for 25(OH)D and $1,25(OH)_2D$ in whole blood. Preparation of the Final Report for the DOD is underway.

Conclusion

Our data indicate that white women in the lowest quartile of $1,25(OH)_2D$ have a 3.8 fold increased risk of breast cancer compared to similar women in the highest quartile. The risk, OR (95% CI) was greater among women over the median age of 54, 5.2 (2.4, 11.1), than for younger women, 1.3 (0.7, 2.6).

Future work needs to address the temporal relationship between low levels of vitamin D metabolites and the development of breast cancer. A case-control study design cannot evaluate the temporal relationship; a prospective study would provide certainty on this question. A replication of this study using the preferred substrate of plasma or serum would allow a more accurate assessment of the $1,25(OH)_2D$ levels which confer risk or protection.

Black women did not demonstrate the same relationship between low levels of $1,25(OH)_2D$ and breast cancer risk. It is important to study a larger group of black women and other minority women to clarify the vitamin D/breast cancer relationship in this population.

The information on VDRs is important; differences in quantity and/or function may be associated with breast cancer risk or prognosis. Further, two recent papers described an association between Taq1 VDR genotype and prostate cancer risk[13,14].

The significance of this work relates to the potential for prevention of breast cancer. Recent work demonstrated the antineoplastic effect of a novel vitamin D analogue, 1α-hydroxyvitamin D_5[15]. Vitamin D analogues are potential chemotherapeutic as well as chemopreventive agents for breast cancer[15]. Finally, there is the possibility of better understanding the mechanisms of carcinogenesis as we define the role of vitamin D in this process.

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